

Genet Composition of *Diphasiastrum complanatum* in Western Hungary: a Case Study

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ABSTRACT.—The study includes an investigation of the genetic composition of a marginal ground pine (*Diphasiastrum complanatum*) population consisting of patches of different size. The genetic analysis was performed on the basis of 15 isozyme loci. The proportion of polymorphic loci was $P=0.466$; the sampled 38 ramets were classified into 21 multilocus genotypes (genets); Pielou's clonal diversity index was $D=0.898$. Wright's fixation indices over polymorphic loci were -0.076 (0.063) and -0.026 (0.091) at the ramet and genet-level, respectively. The observed and expected ramet-level and genet-level average heterozygosities were not significantly different in spite of the fact that at some loci genotype numbers significantly differed from Hardy-Weinberg proportions. Principal coordinates analysis revealed that the genetic composition of the genets was independent in all but one patch, and spatial autocorrelation analysis revealed significant spatial genetic structure. No individual with homozygous genotypes at all loci was observed, indicating a lack of intragametophytic selfing. Results clearly showed that in addition to clonal growth, sexual reproduction played a substantial role in the establishment and maintenance of the study population at the boundary of the species' distribution, and indicated the importance of microsite conditions.

Populations of colonizing and clonal plants are usually genetically depauperate because of bottlenecks and selfing during colonization, and different forms of vegetative reproduction that overwhelm sexual recruitment (Darlington, 1958; Stebbins, 1950). During clone establishment, successfully competing genets occupy all available microhabitats, so populations frequently are assumed to originate from only a small number of founder genets. In addition, homosporous ferns and other pteridophytes are apt to undergo intragametophytic selfing, further decreasing the genetic variability within the population while genetic divergence among populations can increase. In contrast, gene flow into established populations, recombination, plus secondary (repeated) seedling recruitment can result in increased genotypic variability within populations.

Ellstrand and Roose (1987) compiled a summary of 27 studies that revealed a tendency of 20 clonal plant species to form multiclonal populations of intermediate diversity and evenness, and most clones proved to be limited to few populations. Eriksson (1993) summarized the state of the art of genet dynamics in clonal plants, and called attention to the fact that the whole life cycle and genet structure (independent or connected ramets and growth form) must be taken into account to obtain a clear picture of dispersal, recruitment, and the genetic composition and structure of clonal plant populations. More-

over, a particular species can produce different patterns under different conditions, the genet and ramet dynamics can alter in different habitats (Kull, 1995), and the genetic structure of a population partly depends on the history of the site (Eriksson and Bremer, 1993). Therefore, in spite of their extremely long lifespan and considerable size of individual clones, clonal organisms can maintain relatively high genotypic variation that has large differences between populations (Kemperman and Barnes, 1976; Jonsson, 1995; Jonsson et al., 1996). Usually, the role of generative reproduction increases with small scale disturbances (Eriksson, 1989; 1993), and on more stressed sites, where the intragenet competition is lower (Wu et al., 1975).

Concerning the Lycopodiaceae, Levin and Crepet (1973) in their earliest study investigated the genetic variation of *Lycopodium lucidulum* Michx. (*Huperzia lucidula* (Michx.) Trevis.) in 16 New England populations. Individual populations showed little to moderate genetic variation; the proportion of polymorphic loci varied from 5–28%. A low level of variability within the populations frequently was coupled with uniform heterozygosity for the same alleles at some loci, and with regional differentiation. Among the 242 individuals of 16 populations, they found only 19 multilocus genotypes; the clonal diversity ranged 0.14–0.77. This observed low diversity was partly explained by the phylogenetically relic state of the species and the disproportionate balance between asexual and sexual reproduction.

The clonal growth characteristics of the European clubmoss *Lycopodium annotinum* L. were investigated thoroughly in Swedish Lapland (Callaghan et al., 1986a, b; Svensson and Callaghan, 1988a, b; Carlsson et al., 1990). They found that the most important factors in the maintenance of the populations were an opportunistic guerilla growth-form, and long survival of the genets. In his comparative study, Oinonen (1967; 1968) found that the guerilla growth-form was not so obviously expressed in *L. complanatum* (*Diphasiastrum complanatum* (L.) Holub), because the annual growth of the horizontal branches proved to be less intensive than that of *L. annotinum* and *L. clavatum* L. (Lycopodiaceae); moreover, the distribution of the vertical branches was more closely packed.

More recently, several papers have been published on the mating system and genetic structure of other clonal fern species, or have analysed intragametophytic selfing in clonal ferns and in those with subterranean gametophytes (McCauley et al., 1985; Soltis and Soltis, 1986; Holsinger, 1987; Soltis and Soltis, 1987; Soltis and Soltis, 1988a; b; c; Soltis et al., 1988a; b; Wolf et al., 1987; 1988). Soltis and Soltis (1990) published fixation indices ranging from –0.590 to 0.672 for lycopods (*Lycopodium*, *Huperzia*), genetic diploidy (Soltis and Soltis, 1988a), and variable intragametophytic selfing among populations. For *Diphasiastrum complanatum* and *D. digitatum* (A. Braun) Holub, no intragametophytic selfing was reported in spite of their subterranean gametophyte development.

The aim of this study was to investigate the importance of the sexual reproduction, genetic diversity, clonal growth, and intragametophytic selfing within and between the patches of *D. complanatum* in a population. *Diphasiastrum*

complanatum is a very rare, protected species in the Hungarian flora, because it reaches the southern boundary of its distribution in the Carpathian basin. It was an important consideration that the sampling should not damage the relatively small population. The simplest method to detect the genet structure of the population was the application of isozyme analysis. The number of genets in the patches can be revealed by this method, and it is possible to estimate their size, to compare the genetical and topographical distances among them, and to give elementary characterization of the genetic structure of the population.

MATERIALS AND METHODS

STUDY AREA, PLANT MATERIAL, SAMPLING.—This study was carried out at the western boundary of the Carpathian basin in Hungary near Szentgotthárd (“Vendvidék”; latitude 46°53′N, longitude 16°15′E). The bedrock of the area is alluvial drift-boulder, clay, and adobe. The study area is located at 300 m above sea level. The average annual precipitation is approximately 800 mm and the mean annual temperature is 9.1°C. The vegetation is zonal mixed coniferous-deciduous forest (*Genisto nervatae-Pinetum* [Pócs, 1965]) and extrazonal, probably artificial spruce forests. The soil is illuviated brown forest soil with slight podsolization. In most parts of Hungary the zonal vegetation at this altitude is Turkey oak wood (*Quercetum petraeae-cerris*). The appearance of the mixed forest at this low altitude is explained by the vicinity of the Alps and occurrence of nutrient poor acidic soil, as well as by the history of the vegetation (Ódor, 1996).

The sampling was carried out in seven patches of *D. complanatum* in May 1996, for the apices of horizontal branches, and in June 1996, for the young tips of the vertical modules. Voucher specimens are on deposit at the Genetics Department of Eötvös Loránd University. The patches were very different in size and shape (Fig. 1). The sampling points were allocated along the boundary of the patches except G, N, and P. Patch II was sampled more intensively, as patches II and III were denser, whereas the others were looser. Some parts of patch V seemed to be physically isolated. Patch VI was situated in a 20–30 year old planted spruce wood in which shrub, herb, and moss layers were less developed than the vegetation of the other patches, which belonged to similar old stands of a deciduous-coniferous mixed forest with minor difference among them (Ódor, 1996).

ISOZYME ANALYSIS.—1) Sample preparation: For the separation of GOT (glutamate-oxaloacetate transaminase, E. C. 2.6.1.1), EST (colorimetric esterase, E. C. 3.1.1.1), LAP (leucine aminopeptidase, E. C. 3.4.11.1), PER (peroxidase, E. C. 1.11.1.7), PGI (phosphoglucose isomerase, E. C. 5.3.1.9), and ACP (acidic phosphatase, E. C. 3.1.3.2), 200 mg of the actively growing horizontal branches were ground in a chilled mortar in 1 ml of the following extraction buffer: 0.1 M K-phosphate, pH=7.5, 0.029 M Na-tetraborate, 0.017 M K-metabisulfite, 0.2 M L-ascorbic acid, 0.016 M dithiothreitol, 4% soluble PVP, with 2 g sucrose

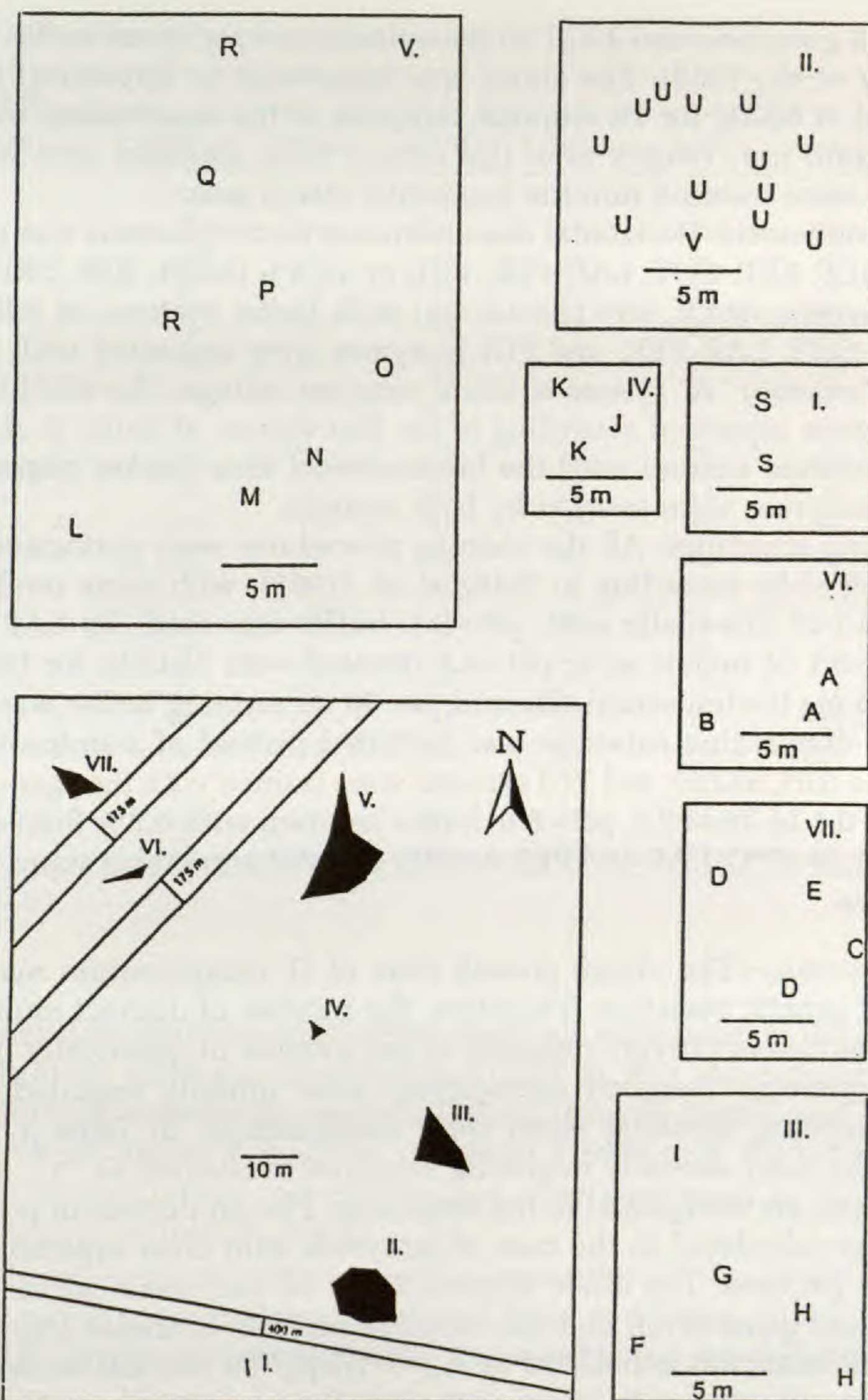


FIG. 1. Bird's-eye view of the location of the patches (bottom left), and the location of the sampled ramets in patches I–VII.

and 10 μ l 2-mercaptoethanol as additives per 10 ml grinding buffer. For the extraction of MDH (malate dehydrogenase, E. C. 1.1.1.37), IDH (isocitrate dehydrogenase, E. C. 1.1.1.42), and SKDH (shikimate dehydrogenase, E. C. 1.1.1.25), 200 mg of the branches were homogenized in a chilled mortar in 1 ml of the following grinding buffer: 0.04 M Tris-HCl, pH=7.8, 0.01 M $MgCl_2$, 0.001 M EDTA, 0.005 M dithiothreitol, 0.013 M K-metabisulfite, 4% soluble

PVP, with 2 g sucrose, and 10 μ l 2-mercaptoethanol per 10 ml buffer (modified after Soltis et al., 1983). The slurry was transferred to Eppendorf tubes and centrifuged at 6000g for 10 minutes. Aliquots of the supernatant were stored at -80°C until use. Twenty μ l of this extract were absorbed onto filter paper wicks that were inserted into the horizontal starch gels.

2) Electrophoresis: Horizontal discontinuous electrophoresis was performed on 11% (ACP, EST, GOT, LAP, PER, PGI) or 11.5% (MDH, IDH, SKDH) starch gels (Connaught starch, $26 \times 12 \times 0.8$ cm) with buffer systems as follows. The ACP, EST, GOT, LAP, PER, and PGI isozymes were separated with Arulsekhar and Parfitt's (1986) "A" system at 130 V constant voltage. The MDH and SKDH isozymes were separated according to the first system of Soltis et al. (1983) at 100 mA constant current until the bromophenol blue marker migrated 7 cm. The IDH isozymes were assayed by both systems.

3) Staining schedules: All the staining procedures were performed on 2mm thick starch slabs according to Soltis et al. (1983), with some modifications. For EST, 0.1 M Tris-malic acid, pH=6.4, buffer was used; for LAP isozymes 0.1 M Tris-0.1 M maleic acid, pH=4.5 (titrated with NaOH); for GOT an additional 10 mg bovine serum albumin per 50 ml staining buffer were applied; for PER O-dianisidine substrate was included instead of 3-amino-9-ethylcarbazole. The IDH, SKDH, and PGI systems were stained with the agar-overlaying method in 0.1 M Tris-HCl, pH=8.0, buffer solution with 0.5% final concentration of agar, at 37°C ; LAP and PER usually showed acceptable staining at room temperature.

DATA ANALYSES.—The clonal growth form of *D. complanatum* may bias the analysis of genetic variation. Therefore, the number of distinct multilocus genotypes was conservatively regarded as the number of genetically distinct individuals (genets). Sampled sporophytes were initially regarded as ramets without knowing anything about their relationships. In Table 1, the locus (zone) of the most anodally migrating isozymes is labelled as "1", and alleles within a zone are designated in the same way. The proportion of polymorphic loci (P) was calculated in the case of isozymes with clear separation and interpretable patterns. The allele frequencies at all loci were calculated for ramet-level and genet-level, and the effective number of alleles (A_e) was determined. The latter was calculated as $A_{ek} = 1/\sum p_{ik}^2$ for the kth locus, where p_{ik} is the frequency of the ith allele of the kth locus, and averaged over loci as A_{eR} and A_{eG} on ramet- and genet-level, respectively. The clonal diversity in the population was characterized with the modified Simpson diversity index (Pielou, 1969): $D = 1 - \sum \{[n_i(n_i - 1)]/[N(N - 1)]\}$, where n_i is the number of the ramets of the ith multilocus genotype and N is the number of the sampled ramets. Furthermore, observed (H_{ok}) and expected genetic diversity ($H_{ek} = 1 - \sum p_{ik}^2$) was averaged over all polymorphic loci for both ramet- and genet-level (H_{oR} , H_{eR} and H_{oG} , H_{eG}). Deviation from Hardy-Weinberg equilibrium at each variable locus was tested by chi-square test. Wright's (1965) fixation indices were calculated for each polymorphic locus, and averaged as F_R and F_G at the ramet-level and genet-level, respectively.

TABLE 1. Genotype of genets at the polymorphic loci and the number of ramets in a genet.

Genotype		MDH-1	MDH-4	SKDH	EST-1	EST-4	PGI-2	PER
Genet	Ramet							
A	2	12	11	11	22	22	33	22
B	1	12	11	11	22	22	22	11
C	1	12	11	11	12	22	22	12
D	2	12	11	23	22	22	23	12
E	1	12	11	23	12	22	23	12
F	1	11	11	11	22	12	22	12
G	1	12	11	11	12	22	23	12
H	2	12	11	13	12	22	23	12
I	1	12	11	22	12	12	22	12
J	1	12	12	22	22	12	23	22
K	2	11	11	11	22	12	23	22
L	1	22	11	23	22	22	23	11
M	1	22	11	23	22	12	22	12
N	1	12	11	11	22	12	22	12
O	1	22	11	23	22	11	22	11
P	1	22	11	23	12	11	12	12
Q	1	11	11	33	22	11	33	12
R	2	12	12	23	22	11	23	12
S	2	11	11	23	22	11	22	11
U	12	12	11	33	22	11	23	22
V	1	12	11	22	22	11	23	22

For further data analyses multivariate methods were used on the basis of the genet multilocus genotype distribution (Table 1). For the ordination of the genets, principal coordinates analysis (PCoA, [Gower, 1966]) was carried out using the chord-distance function of Orlóci (1975) and Euclidean-distance function of Podani (1980). All calculations were performed by the SYN-TAX program package of Podani (1993).

Geographical evenness of the genetic composition of the genets was investigated by spatial autocorrelation analysis (Sokal and Oden, 1978a, b). Wartenberg's SAAP version 4.3 (1989) Spatial Autocorrelation Analysis Program was used to calculate Moran's I coefficient and its statistical significance. Moran's I is a special type of the Pearson product-moment correlation coefficient that describes the deviation of the neighboring points from the mean of all observations. Values of I significantly larger than the expected value $E(I) = -(n-1)^{-1}$ show that the points (individuals) in that distance class are more similar than would be expected by chance, and values significantly lower than $E(I)$ show that individuals are less similar. In both cases some factors are affecting the distribution and not chance alone. All univariate autocorrelograms and the average autocorrelogram were analysed for different sets of distance classes (different numbers of distance classes of equal size or with equal numbers of point pairs).

RESULTS

VARIABILITY OF ISOZYME SYSTEMS.—ACP and LAP enzyme activity could be detected only at a single monomorphic band. GOT isozymes showed a monomorphic zone with strong activity and occasionally a second, faintly stained, more cathodal zone which was unscorable.

EST isozymes migrated into both the cathodal and anodal regions. The bulk of activity appeared in the anodal part where several zones could be distinguished. The separation and activity of isozymes were interpretable and scorable in the fastest zone (EST-1), and in the fourth region (EST-4). There appeared two putatively allelic isozyme forms in both zones. The three-banded pattern of the putative heterozygotes reflects a dimeric esterase coded by locus EST-4. The inheritance of these latter electromorphs should be tested by directed crosses.

PGI isozymes were resolved in two zones probably coded by two loci. The activity in the faster region (PGI-1) was monomorphic but frequently faint and hardly visible. In the slower region (PGI-2), there occurred three allelic forms, with three-banded patterns in putative heterozygotes, indicating a dimeric enzyme (Fig. 2a).

IDH isozymes separated by the method of Soltis et al. (1983) appeared in two zones of the anodal part of the gel, and may most likely be coded by two loci. In the more cathodal region there occurred a monomorphic single band with faint intensity (IDH-2). The more anodal region (IDH-1) showed a three-banded pattern (Fig. 2b). We could observe four (a–d) phenotypes in this region with slight differences in the position of the bands. There may be alternate interpretations for this region. On one hand, there may be a polymorphic locus with four alleles. The minute differences in the mobility of the bands seem to contradict this interpretation. On the other hand, this could be a duplicated locus with homozygosity at the individual loci and an interlocus heterozygote isozyme band. This possibility is indicated by the fact that among the sampled 38 ramets there was not a single individual with the characteristic one-banded homozygote phenotype. According to a third interpretation, the pattern may be produced by a monomorphic enzyme that suffered special modification/degradation processes. We decided on separating these isozymes with the “A” system of Arulsekhar and Parfitt (1986) to check the stability of the patterns. In this case, the phenotypes were quite different: there appeared a monomorphic active isozyme followed by two faint ghost bands (Fig 2c). Therefore, IDH-1 was conservatively interpreted as a monomorphic locus producing multiple banded phenotype originating from secondary modifications or degradation.

MDH isozymes separated in the anodal part of the gel in four different zones. Zones “1” and “4” were variable, probably as independent loci (*mdh-1* and *mdh-4*) with two alleles and three-banded heterozygote patterns (Fig. 2d), whereas in zones “2” and “3” monomorphic activity was detected. These latter may be two independent loci (*mdh-2* and *mdh-3*).

PER isozymes showed very complex and different patterns in the vertical

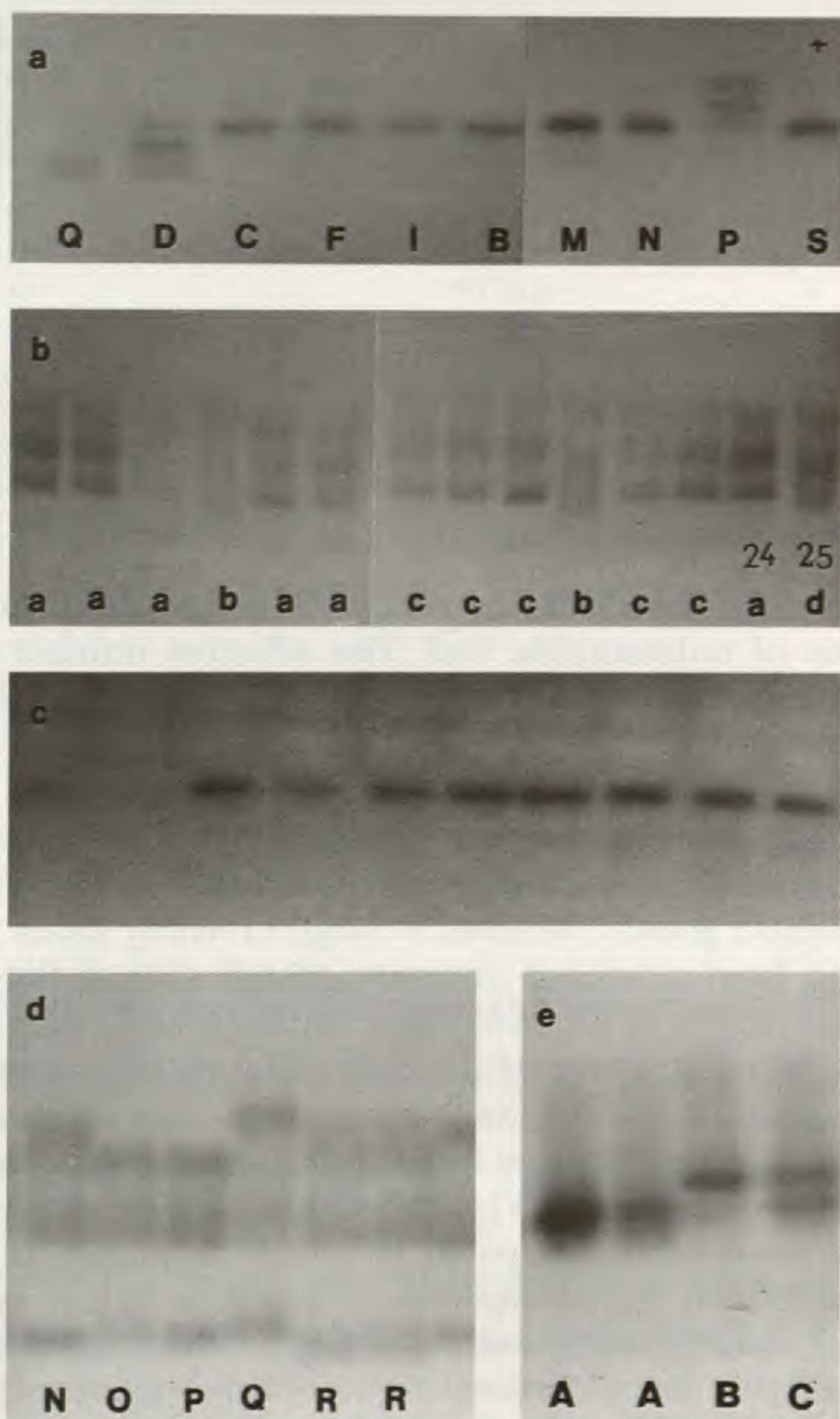


FIG. 2. Patterns of some isozymes of *Diphasiastrum complanatum*. a) PGI-2 isozymes of genets Q, D, C, F, I, B, M, N, P, and S. b) IDH-1 isozymes separated with system 1 of Soltis et al. (1983); letters a–d show the variants detected only by this system. c) IDH-1 isozymes separated with Arulsekhar and Parfitt's (1986) system A; all the ramets produced the same pattern; light bands of SOD (superoxide dismutase, E. C. 1.15.1.1) isozymes are visible more anodal from the IDH-1 activity. d) MDH isozymes of genets N, O, P, Q, and R. e) PER isozymes of young green vertical branches of genets A, B, and C. See Table 1 for detailed genotyping at the individual loci.

green branches and the underground horizontally growing branches. In the underground branches, both the cathodal and the anodal isozymes showed high activity, but in the green vertical branches the activity of the cathodal isozymes decreased, and the resolution was unsatisfactory. The most intensively stained anodal region could be interpreted as a putative locus with two allelic forms (Fig. 2e).

SKDH activity appeared in a single zone of the anodal part, near the origin. The pattern was interpretable as a locus with three allelic electromorphs.

TABLE 2. F indices of polymorphic loci. (Standard deviations of means are in parentheses). Significant difference between expected and observed genotype numbers. * P < 0.05; ** P < 0.01; *** P < 0.001.

Locus	Ramet-level:	Genet-level:
EST-1	-0.102	-0.167
EST-4	-0.102	-0.167
MDH-1	-0.477**	-0.238
MDH-4	-0.040	-0.282
PER	0.106	-0.145
PGI-2	-0.373*	-0.103
SKDH	0.456***	0.356***
Means (S.D.)	FR = -0.076 (0.063)	FG = -0.026 (0.091)

Out of the investigated 15 loci 7 proved to be polymorphic giving $P = 0.467$ as the proportion of polymorphic loci. The effective number of alleles (and standard deviation) was $A_{eR} = 1.33$ (0.14) and $A_{eG} = 1.38$ (0.15) for ramet- and genet-level, respectively.

Because there was no ramet with a homozygous genotype at all loci, no intragametophytic selfing was detected. Among the 38 investigated ramets there were 21 different multilocus genotypes (Table 1, Fig. 1) that could be considered as distinct genets. Simpson's clonal diversity index was $D = 0.898$, indicating a high level of clonal diversity: 66.6% (14) of the 21 genets was present as unique ramets in the samples, a further 28.6% (6) was present only in 2 ramets. The genet structure of the individual patches showed substantial variability. The smallest patch I contained a single genet (S), although its ramets (24 and 25) showed a minute difference in their IDH-1 pattern, probably by modification or degradation (Fig. 2b). Other smaller patches consisted of different genets: three ramets of patch IV and VI belonged to 2 genets each; four ramets in patch VII were from three genets, five ramets in patch III were from four genets, and the eight ramets of patch V belonged to seven genets. On the contrary, 12 of the 13 ramets in patch II belonged to the same genet, showing the essentially different structure of that patch.

Genetic diversity was calculated on the ramet- and genet-level. Ramet-level average observed heterozygosity was $H_{oR} = 0.174$ (0.066) and the average expected heterozygosity was $H_{eR} = 0.165$ (0.059), whereas genet-level values were $H_{oG} = 0.187$ (0.062) and $H_{eG} = 0.184$ (0.062). The difference between these mean observed and expected heterozygosities was not significant ($t = 0.1$, $df = 3$, $P > 0.05$). Fixation index (F) for each polymorphic locus was estimated and averaged over loci for both ramet- and genet-level (Table 2). Ramet-level averaged at $F_R = -0.076$ (0.063), genet-level at $F_G = -0.026$ (0.091), with no significant deviation between them ($t = 0.17$, $df = 6$, $P > 0.05$). Slight heterozygote excess (negative F value) was detected at five of seven loci on both ramet-level and genet-level, heterozygote deficiency (positive F value) at two loci, but the sign of deviations was not uniform for MDH-4 and PER. For SKDH, a highly significant heterozygote deficiency was indicated in both cases. The chi-square test of observed versus expected genotype numbers showed significant deviation from Hardy-Weinberg proportions at ra-

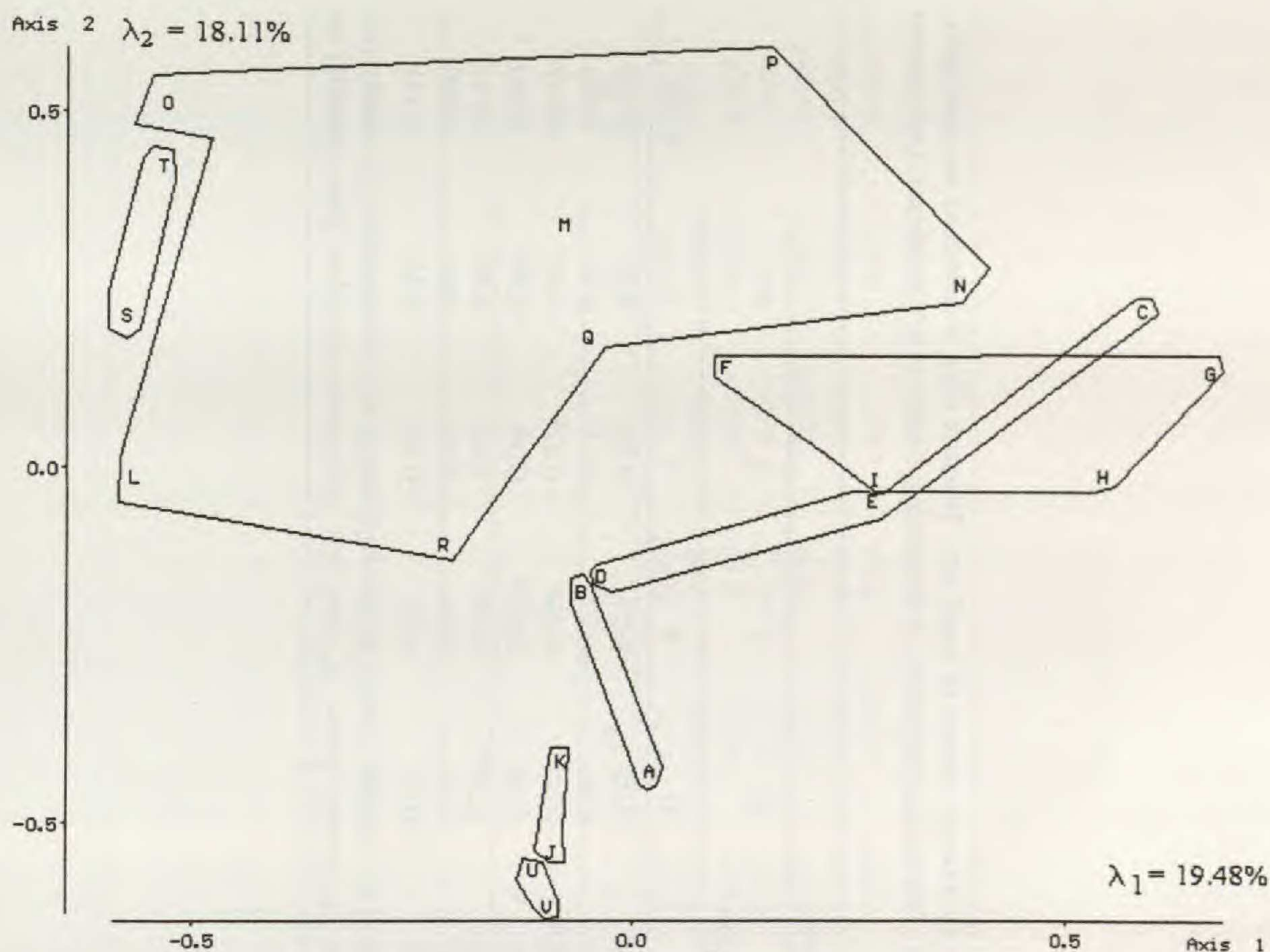


FIG. 3. Principal coordinates analysis of multilocus genotypes based on their isozyme patterns. Ordination was performed on the basis of chord-distance function of Orlóci (1975). Symbols of the genets growing in the same patch are bordered. λ_1 and λ_2 mean the Eigen-values as percentages.

met-level in the case of MDH-1 ($P < 0.01$), SKDH ($P < 0.001$), and PGI-2 ($P < 0.05$), but at genet-level only in the instance of SKDH ($P < 0.001$).

The result of principal coordinates analysis based on the chord-distance function of Orlóci is presented in Fig. 3. Axes I and II together contained 39.4% of the variation. The same ordination was produced with the Euclidean-distance function. Both distance functions ordinated most of the genets as independent of their geographical position. Only genet pairs G–H, L–Q, M–O, and U–V were genetically more related according to their ordination and belonged to the same patches.

Spatial autocorrelation coefficients were calculated with different sets of distance classes. The values of univariate (one locus) autocorrelation coefficients and the average I indices are shown in Table 3a and 3b for the ten distance classes of equal size and equal numbers of point pairs in ten distance classes options, respectively. In some distance classes for some loci there appeared univariate Moran coefficients that significantly differed from those for independent spatial structure. EST-4 and SKDH appeared among these isozymes in both analyses. However, the average I indices definitely detected no significant spatial substructure in the population: $I = -0.001$ (0.002) for the equal size distance classes and $I = -0.050$ (0.033) for the latter case. The average

TABLE 3A. Moran's I (spatial autocorrelation) coefficients of polymorphic loci in 10 distance classes of equal size. Distance class 9 contained no point pairs. Significant deviations from the expected values are labelled * $P < 0.05$; ** $P < 0.01$. The standard deviation of the average I index is in parentheses. Last column shows the significance of the correlograms (C. Pr.).

	D. class									C. Pr.
	1	2	3	4	5	6	7	8	10	
	No. of pairs									
	104	5	24	12	15	41	4	2	3	
MDH-1	-0.05	0.01	-0.15	0.02	-0.36	0.05	-0.51	0.29	0.29	0.710
MDH-4	-0.06	-0.34	-0.08	0.11	-0.12	0.00	-0.17	0.11	0.11	1.000
SKDH	-0.11	1.20**	-0.24	0.11	-0.02	-0.02	0.46*	-0.62*	-0.02	0.012*
EST-1	-0.06	0.12	0.05	-0.30	-0.25	0.06	0.05	0.04	-0.53	1.000
EST-4	-0.02	0.40	-0.07	-0.07	-0.21	0.35**	0.40	0.40	0.40	0.036*
PGI-2	-0.06	-0.01	-0.13	-0.11	0.02	0.01	0.04	-0.30	0.03	1.000
PER	-0.03	-0.89*	-0.08	0.14	0.28	-0.21	0.41	-0.38	0.36	0.191
AVERAGE	-0.06	0.07	-0.10	0.01	-0.03	-0.07	0.10	-0.02	0.09	-0.001 (0.002)

TABLE 3B. Moran's I (spatial autocorrelation) coefficients of polymorphic loci in 10 distance classes of equal size. Significant deviations from the expected values are labelled * $P < 0.05$; ** $P < 0.01$; *** $P \leq 0.001$. The standard deviation of the average I index is in parentheses. Last column shows the significance of the correlograms (C. Pr.).

	D. class										C. Pr.
	1	2	3	4	5	6	7	8	9	10	
	No. of pairs										
	21	21	21	21	21	21	21	21	21	21	
MDH-1	0.70**	−0.09	−0.01	−0.47*	−0.36*	−0.16	0.11	−0.40*	0.24	−0.05	0.001***
MDH-4	−0.05	−0.26	0.13	0.00	−0.11	−0.11	−0.05	0.00	−0.05	0.00	0.911
SKDH	0.24	−0.34	0.02	−0.08	−0.21	−0.27	0.02	0.18	−0.11	0.00	0.658
EST-1	0.03	0.17	−0.27	−0.30	0.07	−0.07	−0.13	−0.13	0.10	0.03	0.968
EST-4	0.17	0.20	−0.20	−0.13	−0.13	0.27*	−0.27	0.00	−0.53**	0.13	0.039*
PGI-2	−0.25	−0.09	0.00	0.02	0.01	−0.16	−0.09	0.05	0.02	−0.01	1.000
PER	0.48*	0.23	−0.93**	0.85**	−0.37*	−0.50**	0.34**	0.14	−0.11	−0.16	0.000***
AVERAGE	0.19	−0.09	−0.18	−0.02	−0.16	−0.14	−0.01	−0.03	−0.06	0.00	−0.050 (0.033)

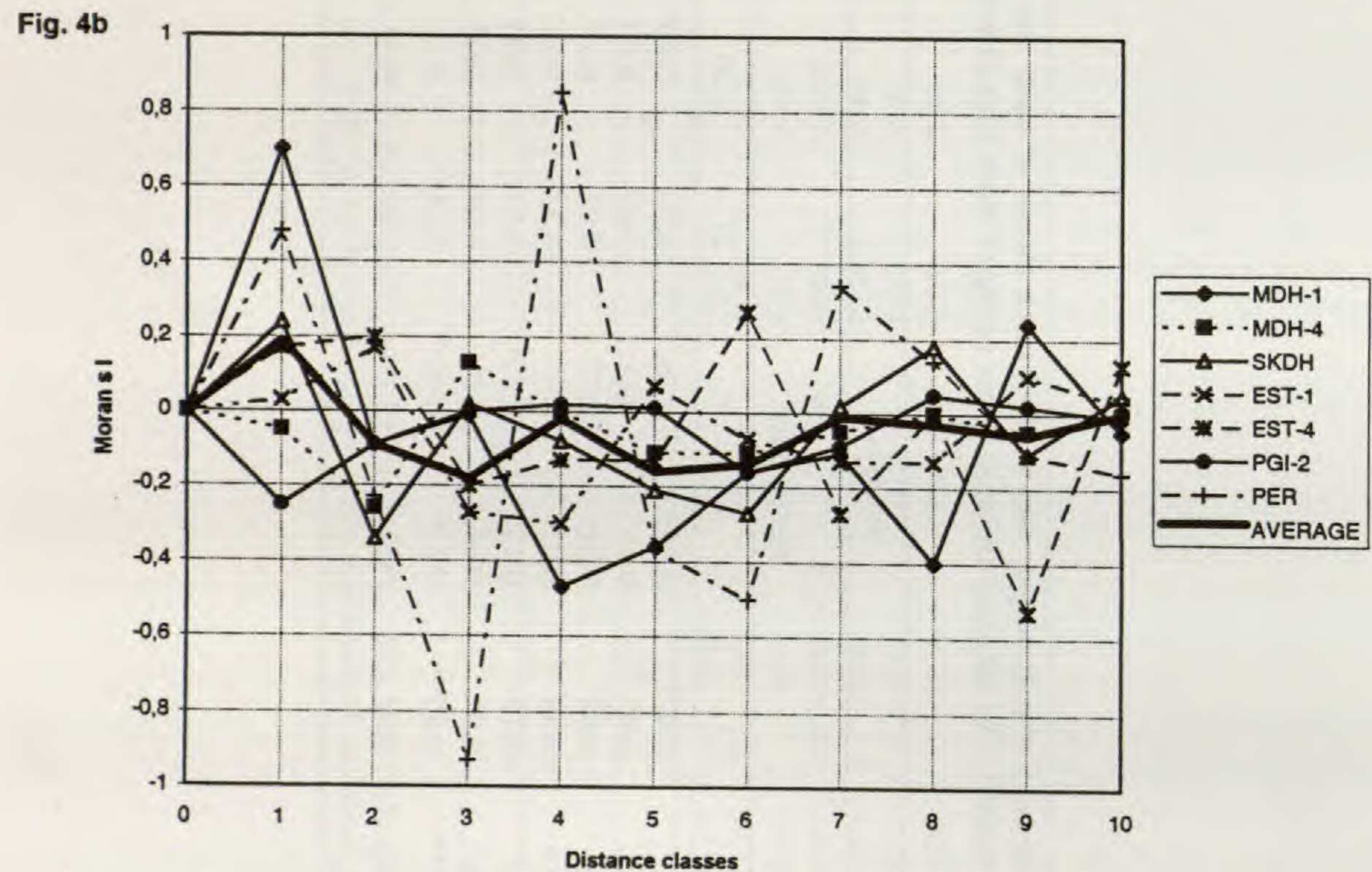
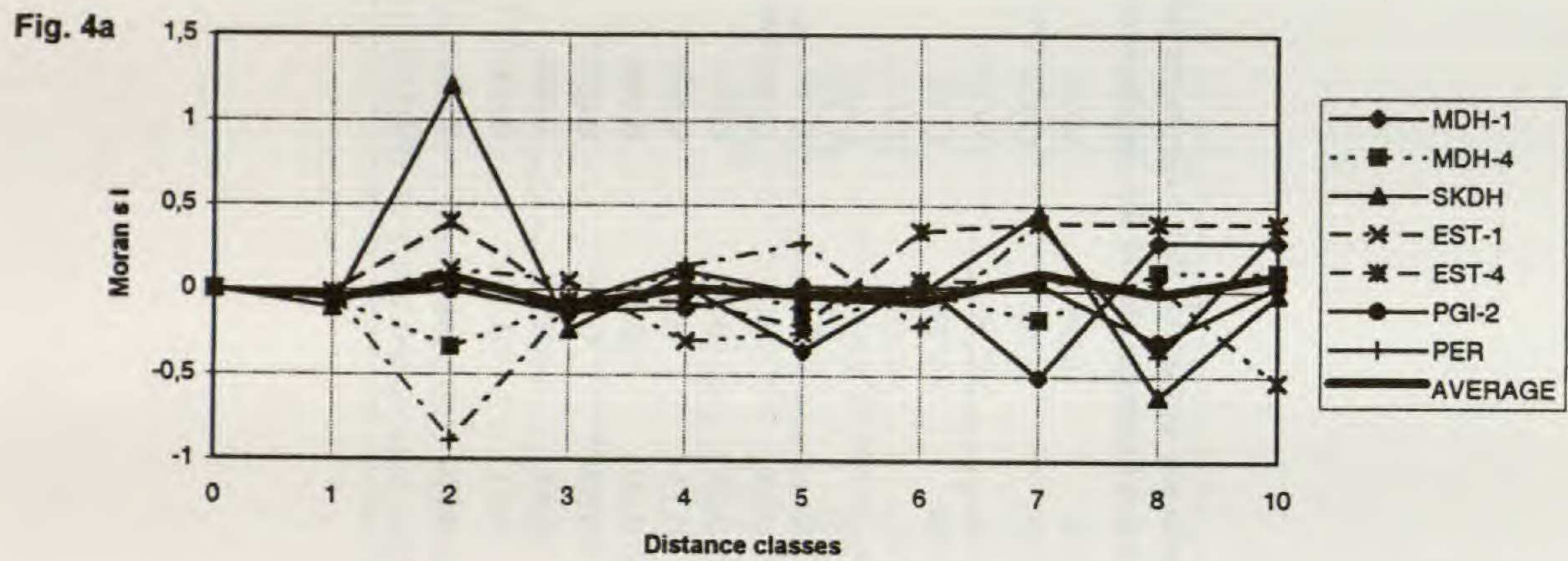


FIG. 4. Spatial autocorrelation analysis of the genet multilocus genotypes. Moran's I autocorrelation coefficients are shown for individual polymorphic loci, and their average is presented in each distance class. a) Correlograms for ten distance classes of equal size (data from Table 3a); distance class 9 contained no point pairs. b) Correlograms for ten distance classes with equal numbers of point pairs in each (Data from Table 3b).

autocorrelograms show a random series of nonsignificant values for all distance classes (Fig. 4a and 4b). The diagrams obtained by other options were similar.

DISCUSSION

The observed numbers of isozymes were in accordance with the data for eight lycopods reported by Soltis and Soltis (1988a) for those enzymes in common in the two studies (GOT, LAP, PGI, and SKDH). In addition to these re-

sults, we managed to visualize ACP, (NADP) IDH, and MDH isozymes of *D. complanatum*, detected two loci for colorimetric esterase (EST), one of them with a dimeric enzyme, and found a locus of PER. Two loci for IDH and four loci for MDH are the numbers usually detected for diploid seed plants, further supporting the concept of genetic diploidy of lycopods. In addition, with the first electrophoresis buffer system IDH-1 isozymes behaved similarly to that was observed by Hickey for *Isoetes* (cited by Duff and Evans [1992] as "Hickey, personal communication"). They explained a fixed uniform three-banded pattern as the product of a monomorphic locus after posttranslational modification or degradation. Actually, in the case of *D. complanatum* the second separation system proved the monomorphic state of the IDH-1 isozymes, but the pattern with the first system was not uniform; there appeared reproducible minute mobility differences between the bands even for two ramets classified as the members of the same clone on the basis of the other isozymes, i.e., ramet 24 and 25 of genet S. These differences can be based on a genetically variable modifier system similar to that suggested by Hickey et al. (1989) for TPI zone III of *Isoetes* species or can show different stages of the same modification/degradation process. Because the background of these alterations could not be clarified, we did not take into account this heterogeneity when evaluating diversity. On the other hand, it would be worth investigating the genetic background of this modification process. Its presence or absence might even have phylogenetic aspects like the TPI modification proteases of some *Isoetes* species.

The high number of unique multilocus genotypes in the *D. complanatum* population studied parallels the results of Ellstrand and Roose (1987). In the review of the genotypic diversity of clonal plants they indicate that these species are prone to having multiclonal populations of intermediate variability and unique genotypic composition. They found that out of 238 populations only 17 consisted of a single genotype; that is, 93% of the surveyed populations were multiclonal. The 17 uniclonal populations belonged to as few as two uniclonal species. At the same time, Ellstrand and Roose (1987) demonstrated that the number and frequency of the genotypes detected were dependent upon the number of characters included in the study, so the estimation of genetic diversity must have been biased. In spite of the fact that in our case the number of characters investigated was not large (15 isozyme loci), we hope that because the sample size exceeded Holsinger's (1987) recommendation of 25 and we detected a relatively high ratio of polymorphic loci, the estimation of the genet number and diversity was not overestimated, and we reliably registered the lack of intragametophytic selfing. Including this study, three observations have been reported about intragametophytic selfing in *D. complanatum*, and none of them revealed any. Soltis and Soltis (1990) found no intragametophytic selfing in two populations examining 17 isozyme loci.

The analyzed population showed considerable genetic variability, with a 0.898 clonal diversity value, 1.81 (0.519) ramet/genet ratio, and 46.7% polymorphic loci, comparable with the average value (39.9%) reported by Hamrick and Godt (1989) for long-lived herbaceous perennials. The observed relatively

high P value and the genetic diversity measures of the present study may characterize the genetic composition of the species, as it is a widespread club-moss. In their review of genetic differentiation in homosporous ferns, Soltis and Soltis (1990) reported average F indices for different lycopods from -0.141 to 0.016 on the basis of 14 population analyses. The -0.076 ramet-level and -0.026 genet-level values observed in this study fit in with their series. The lack of intragametophytic selfing among the 21 genets, the slight tendency to heterozygote excess, and the fluctuation of the F values among loci (on the ramet-level -0.477 to 0.456 and on the genet-level -0.238 to 0.356 , with significant heterozygote deficiency at some loci or excess of heterozygotes at another locus) not only clearly show the importance of sexual reproduction but call attention to the fact that microsite conditions may have had strong effects on the population. In addition, the data might reflect founder effect. Thus, high clonal diversity and the structure of this small marginal population may be the consequence of the history of the population and the site. The relatively large and probably old patch II was practically formed by one clone, only a single ramet belonged to a distinct but genetically close genet that differed in its SKDH genotype. Other patches, like patch V, which had a similar size and was about 100 m from patch II, showed high clonal variation: 7 genets per 8 ramets. This pattern shows that under microsite conditions sexual reproduction must have played an important role. Principal coordinates analysis visually demonstrates that the genetic distance of the genets is independent of their position in the population. As distant genet pairs as M-S (nearly 460 m) and B-N (nearly 200 m) are genetically closely related according to the ordination, whereas genet pairs A-B and J-K in small patches (diameter about 6 m) are independently ordinated. Only four genet pairs listed in the Results were genetically related and belonged to common patches. Spatial autocorrelation analysis, supporting the results of the principal coordinates analysis, did not reveal any trend in the distribution of genetical characters. Irregular significant difference from the value expected for random structure might have occurred because of the fact that the above mentioned genet pairs were related and might be the consequence of the small number of data.

The type of recruitment, which is of primary importance for the substructuring of the population, was not recognizable because the development of gametophytes and young sporophytes could not be observed. Although the vegetation of the patches seemed to be superficially similar, it could have been more heterogeneous from the "plant's point of view" resulting in different patterns in the patches. Moreover, the coenological and ecological conditions are not favorable at the boundary of the area of *D. complanatum* (Ódor, 1996, 1997). The presence of the strongly competitive plants of the deciduous forest could have increased the clonal variation.

Bruce and Beitel (1979) published their observations on a gametophyte community of six lycopods growing in a 30 year old artificial jack pine (*Pinus banksiana* Lamb.) plantation. One of the most important characteristics was the clustering of gametophytes for all species but one. The most spectacular case was *L. lucidulum*: all of the observed 125 gametophytes grew in a single square 45

cm on a side. This clustering is also a common feature of pteridophytes with superficial gametophytes, and clearly indicates the strong controlling effects of the microenvironment. Spore dispersal presumably is not a limiting factor of initialization because of abundant spore production. Meusel and Hemmerling's (1968) and Oinonen's (1968) observations support these findings. They reported that even different clubmoss species could frequently be found in mixed patches, and often not only along the area boundary, but even in the Alps, although their ecological claims were different. As a further consequence, the presence and proximity of gametophytes originating from different sporophytes can increase the chance of outcrossing, which can produce a genetically variable population, maintain genetic diversity, and explain the lack of intragametophytic selfing even at species with subterranean gametophytes.

The presence of the endophytic symbiont fungi is crucial to the survival and development of the prothalli (Freeberg and Wetmore, 1957; Freeberg, 1962), so theoretically it must be a limiting factor of initial colonization and recruitment, and may cause the patchy appearance of the gametophytes. It may also be supposed that the presence of older sporophytes or prothalli can increase the concentration of fungi in the local soil so that it has a positive effect on the recruitment of new genets sustaining diversity.

According to Oinonen's (1967) observations, "Young plants of ground pine start to develop at a normal rate about 4–6 years after they have penetrated the soil surface, and manifestation occurs about . . . 8–20 years after the spores were sown." Therefore, during the early period of the population establishment or under frequent local disturbances and recolonization, genetic diversity can be substantial, but later the number of genets will decrease as a consequence of competition. Despite of the supposed facilitating effect on repeated recruitment the genet dynamics of established populations cannot be intensive because of the slow development of *D. complanatum*. New genets from repeated recruitment are likely to appear around the edges of the areas occupied by established old clones. Considering these results, the patches of the analysed population can represent different colonization states. The unique small genet V of patch II may be a genetically related genet that is a "loser" in a long competition, or the case of a repeated recruitment inserted near the verge of the spot among the ramets of genet U. In the studied *D. complanatum* population, the patches may show a younger successional state of colonization or a permanently stressed condition, except patch II, in which colonization must have been earlier.

According to these findings the genetic diversity may be substantial even in the case of a small marginal *D. complanatum* population. In the early phase, the genetic composition mainly depends on sexual reproduction, then local conditions, history, and age influence the genetic structure of the population. Clonal growth plays more important role in established populations.

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